# Mitochondrial DNA polymorphism among *Rana ridibunda, Rana lessonae* and *Rana* kl. *esculenta* : preliminary study

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ABSTRACT. - *The digest profiles of mitochondrial DNA from individuals belonging to* Rana ridibunda, Rana lessonae *and* Rana *kl.* esculenta *of various origins have been compared using three restriction enzymes* (BamHI, EcoRI *and* PstI). *This allowed to establish the existence of intraindividual length heterogeneity in the three forms and to define five different cleavage maps. One of these maps* (LC) *is only present in frogs from Chevannes, France. Two others* (RP1 *and* RP2), *rather different, coexist in a* R. ridibunda *population from Austria and the last two* (LA1 *and* LA2) *in* R. lessonae *from Apetlon, Austria. The* LA1 *and* LA2 *morphs are also present in* R. *kl.* esculenta *from Apetlon, Austria, as is one* (RP1) *of the morphs found in* R. ridibunda, *although this last species lives far away. The morph* LA1 *seems similar to the morph B observed by SPOLSKY and UZZELL only in* R. ridibunda. *The results of the present study indicate that the relationships of mtDNA between the green frogs are more complex than at first thought.*

## INTRODUCTION

Numerous studies have recently been devoted to the evolutionary problems raised by the European water frogs or green frogs (*Rana* kl. *esculenta* group) (see reviews and discussions for example in HOTZ, 1974; BERGER, 1977; DUBOIS, 1977, 1982; DUBOIS & GÜNTHER, 1982; UZZELL, 1982 a-b; GÜNTHER, 1983). It appears now that *Rana ridibunda* and *Rana lessonae*, abundant in many parts of Central Europe, were probably separated from each other and diverged a few millions years ago (UZZELL, 1978, 1982 a). Since then, they have regained contact, probably on several occasions, and given birth by hybridization to a third form of green frogs, known as *R. esculenta* (BERGER, 1968; DUBOIS, 1977) which reproduces by hybridogenesis (SCHULTZ, 1968; TUNNER, 1974) and which lives nowaday in sympatry with and mates with one or the other parental species, more often with *Rana lessonae* (L-E system of UZZELL & BERGER, 1975; l-e-P, e-l-P systems of GÜNTHER, 1983). DUBOIS & GÜNTHER (1982) recently proposed to consider such hybrid not as species but as belonging to a special systematic category (klepton) (hence designated *Rana* kl. *esculenta*). Others still use the traditional name *R. esculenta* for this form, which they view merely as a "particular case" within the general category of species (UZZELL, 1982 b).

Since mitochondrial DNA (mtDNA) is a convenient marker for studying matriarchal relationships between species (AVISE & LANSMAN, 1983), a survey of mtDNA of the two parental species and their hybrid may enhance understanding of this complex. SPOLSKY & UZZELL (1984) suggested the possibility of an introgressive process from mtDNA of *Rana lessonae* into *Rana ridibunda* via the hybridogenetic hybrid.

This paper presents preliminary results on the comparative analysis of mtDNA of water frogs from France and Austria belonging to all three taxa.

Besides the intraindividual length variability, previously described for *Rana* kl. *esculenta* (MONNEROT, MOUNOLOU & SOLIGNAC, 1984) and observed here also for *Rana lessonae* and *Rana ridibunda* mtDNA we have detected a noticeable variation of the restriction enzyme sites within the two studied populations from Austria whereas there is none among the frogs of the population from France. The comparison of the cleavage maps leads to the recognition of five different types of mtDNA.

## MATERIAL AND METHOD

### MATERIALS

Samples from three different populations were examined.

(1) Chevannes (Yonne, France): 6 *Rana lessonae*, 12 *Rana* kl. *esculenta* (from a mixed *Rana lessonae* + *Rana* kl. *esculenta* population).

(2) Apetlon (Burgenland, Austria): 18 *Rana lessonae*, 10 *Rana* kl. *esculenta* (from a mixed *Rana lessonae* + *Rana* kl. *esculenta* population).

(3) Prellenkirchen (Lower-Austria, Austria): 4 *Rana ridibunda* (from a pure *Rana ridibunda* population).

### mtDNA PREPARATION

Frogs were analysed one by one. The mitochondria of each individual were isolated from ovaries according to CALLEN et al. (1983) and their mtDNA was purified on CsCl gradients.

### mtDNA COMPARISON

The mtDNA comparison was achieved in two steps. First, mtDNA from individuals belonging to the three taxa digested by three enzymes: *Bam*HI, *Eco*RI and *Pst*I. For each restriction enzyme, the mtDNA digests were compared within each population of each species. Second, a migration, side by side, of samples representative of each type of profiles allowed precise identification of the restriction fragments with similar mobility. Their identity was verified by cross-hybridization (after transfer onto NEN membrane) with $^{32}P$ labelled plasmid DNAs carrying defined amphibian mtDNA genes as described by MONNEROT, MOUNOLOU & SOLIGNAC (1984). Routinely 10 to 100 ng mtDNA per slot (depending on the enzyme chosen) were used, digestions being achieved according to the supplier instructions (BOEHRINGER). Electrophoresis was performed on 1 % vertical agarose gels, overnight, followed by transfer onto Gene-Screen membrane and hybridization with $^{32}P$-labelled *Rana* kl. *esculenta* mtDNA. The smallest fragments detected by this technic were about 300-500 bp long.

### RESTRICTION MAPPING

With the cleavage map of *Rana* kl. *esculenta* mtDNA already in hand

(MONNEROT, MOUNOLOU & SOLIGNAC, 1984) the comparison of the digest profiles of all the mtDNAs allowed to draw their respective cleavage maps. The homology between fragments with different mobility was also insured through the cross-hybridizations done for mtDNA comparison (see above).

## RESULTS

Mitochondrial DNAs from individuals belonging to the three taxa *Rana lessonae*, *Rana ridibunda* and *Rana* kl. *esculenta* of different geographic origin have been digested by three enzymes: *Bam*HI, *Eco*RI and *Pst*I.

At first it has to be pointed out that the intraindividual length heterogeneity described for *Rana* kl. *esculenta* (MONNEROT, MOUNOLOU & SOLIGNAC, 1984) is also observed for *Rana lessonae* and *Rana ridibunda* (fig. 1, 2, 3) with the same properties – i.e. each animal is heteroplasmic and the molecules it carries can be characterized by the range of their variation (400-700 bp) and the extreme sizes. The extensive length variability will be

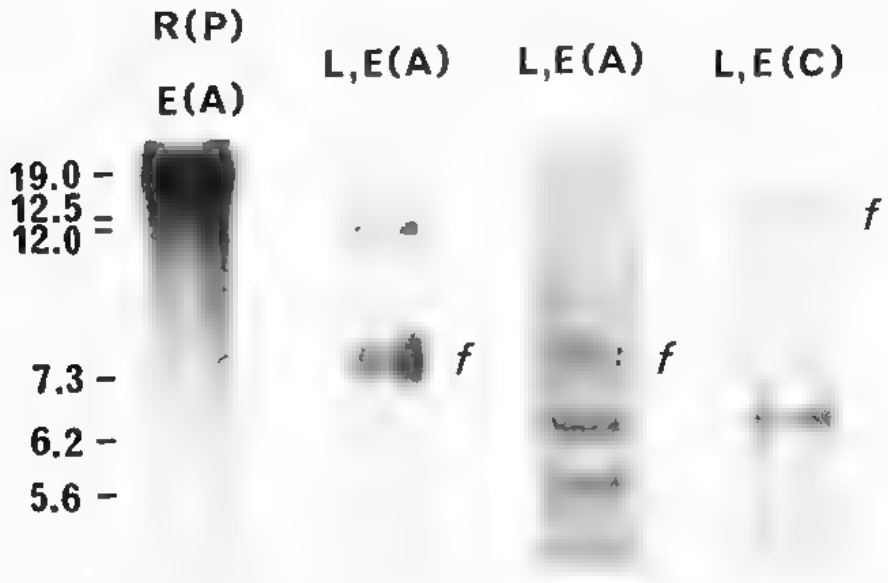


Fig. 1. - *Bam*HI digest profiles of mitochondrial DNA from *Rana ridibunda* (R), *Rana lessonae* (L) and *Rana* kl. *esculenta* (E) of various origins: P = Prellenkirchen (Austria), A = Apetlon (Austria), C = Chevannes (France). The fragments noted (f) originate from the length variable region of the genome.

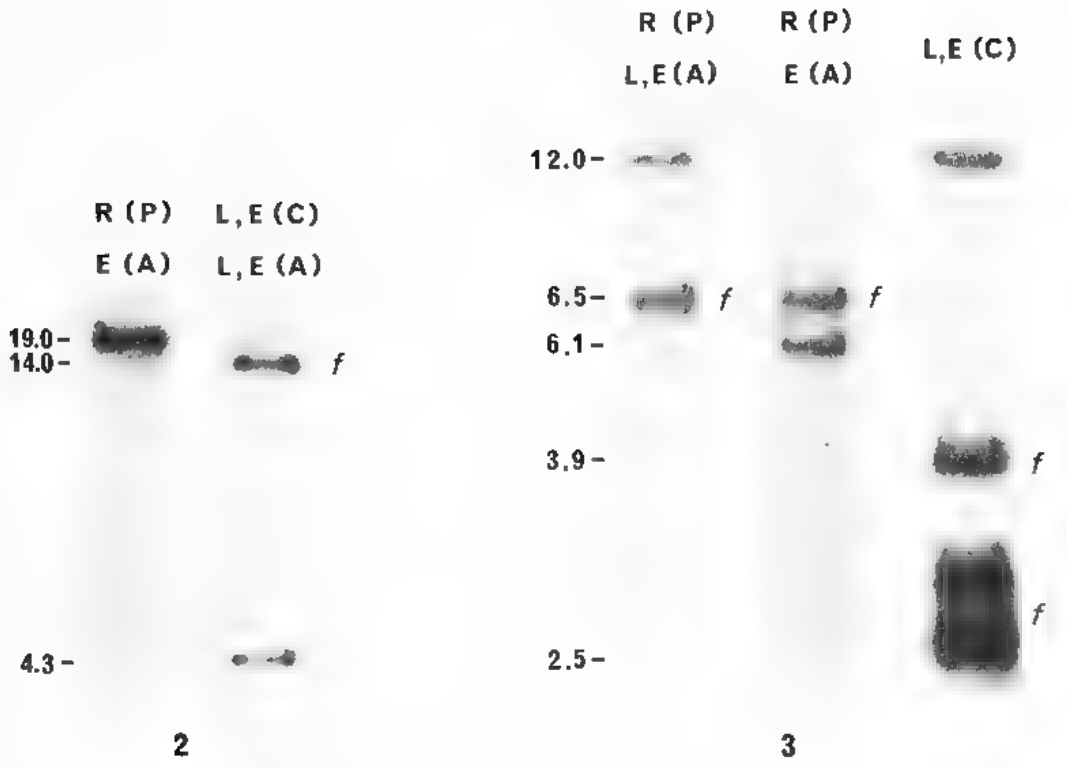


Fig. 2. - *Eco*RI digest profiles of mitochondrial DNA from *Rana ridibunda* (R), *Rana lessonae* (L) and *Rana* kl. *esculenta* (E) of various origins.

Fig. 3. - *Pst*I digest profiles of mitochondrial DNA from *Rana ridibunda* (R), *Rana lessonae* (L) and *Rana* kl. *esculenta* (E) of various origins: see fig. 1.

the subject of another paper; the present analysis of the mtDNA polymorphism will only take the restriction sites into account.

## COMPARISON OF mtDNAs OF *RANA LESSONAE* AND *RANA* KL. *ESCULENTA* FROM FRANCE

No site variation has been detected among mtDNAs of *Rana* kl. *esculenta* and *Rana lessonae* from Chevannes (12 and 6 individuals respectively) (fig. 1, 2, 3).

COMPARISON OF mtDNAs OF *RANA LESSONAE*, *RANA RIDIBUNDA* AND RANA KL. *ESCULENTA* FROM AUSTRIA

On the contrary to the precedent situation the mtDNAs of the frogs from Austria are diversified. For *Bam*HI, there can be observed three profiles (fig. 1): two are found in *Rana lessonae*, the third in *Rana ridibunda*. *Rana* kl. *esculenta* reveals all three profiles. For *Eco*RI, two profiles appear: one is seen in all *Rana lessonae*, the other in *Rana ridibunda* and both in *Rana* kl. *esculenta* (fig. 2). For *Pst*I, two profiles are identified: both are seen in *Rana ridibunda* as well as in *Rana* kl. *esculenta*, but only one has been detected in *Rana lessonae* (fig. 3).

COMPARISON OF THE WHOLE SET OF mtDNAs

In a composite comparison of the various DNAs all these profiles are integrated in terms of cleavage maps. This is done enzyme per enzyme in fig. 4 and the synthesis is presented in fig. 5. Thus we have drawn the

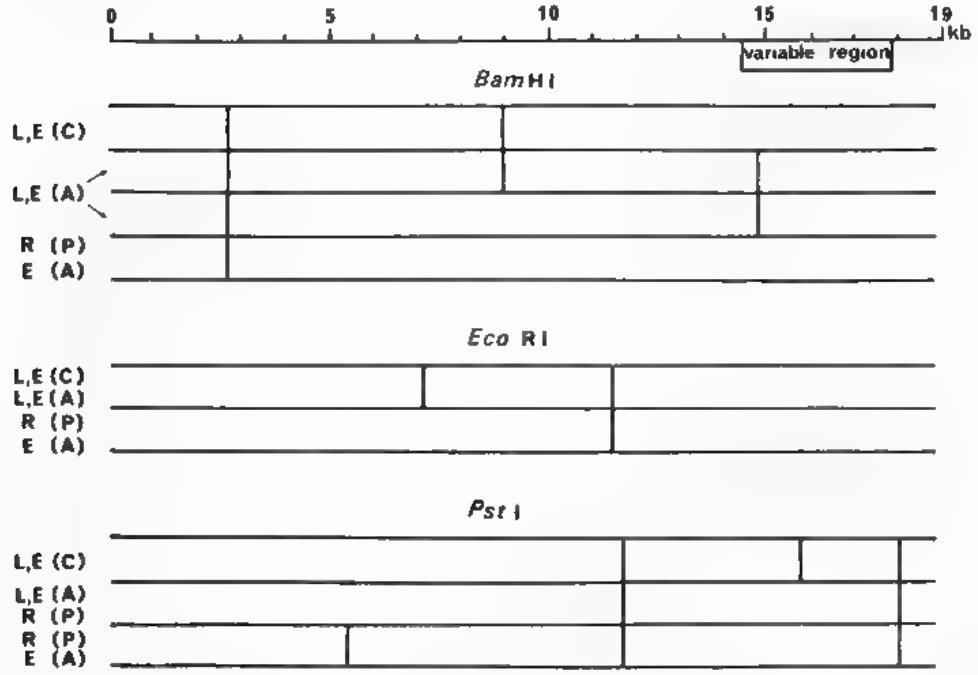


Fig. 4. - Comparison of the different *Bam*HI, *Eco*RI and *Pst*I restriction maps. The homologous sites have been placed in front of each other. For the abbreviations see legend of fig. 1.

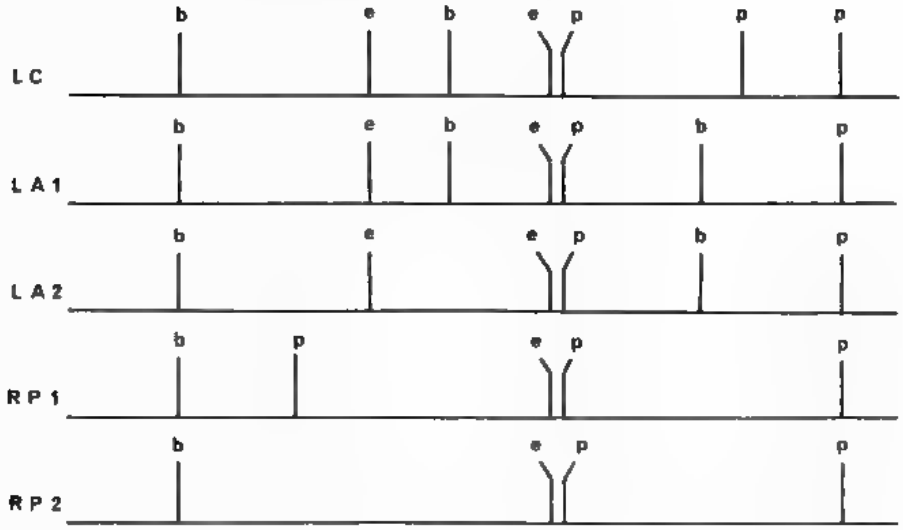


Fig. 5. - Different types of mitochondrial DNA cleavage maps. b = *Bam*HI, e = *Eco*RI, p = *Pst*I. Each type is named according to the form to which it belongs (L = *Rana lessonae*, R = *Rana ridibunda*), to the geographic origin of the population (C = Chevannes, A = Apetlon, P = Prellenkirchen) and numbered in the order of its discovery, if necessary.

cleavage maps of the different mtDNAs observed in the overall study. Each mtDNA-morph has been named according to the species to which it belongs (L: *R. lessonae*; R: *R. ridibunda*), to the geographic origin of the population (C: Chevannes; A: Apetlon; P: Prellenkirchen) and numbered following its discovery, if necessary.

The following information emerge from the overall comparison of these nucleomorphs.

(a) *mtDNA-morph LC*: This morph is specific to the animals from Chevannes. All frogs share the same morph, whatever the taxon, *Rana lessonae* or *Rana* kl. *esculenta*. Remarkably, this morph has not been found in the studied animals from Austria.

(b) *mtDNA-morphs LA1 and LA2*: These two morphs can be distinguished within the *Rana lessonae* from Apetlon, with apparent different frequency (LA1 : LA2 = 14 : 4). They differ by one site (*Bam*HI) over the 7 mapped. Both morphs have also been found in four (respectively two and two)

of the ten hybrid frogs (*Rana* kl. *esculenta*) from Apetlon.

(c) *mtDNA-morphs RP1 and RP2*: Both characterize *Rana ridibunda*. These morphs are rather different from all other mtDNAs we have studied so far (4 sites in common with LA1 over the 7 identified). It seems most interesting that the morph RP1, seen in the pure *Rana ridibunda* population of Prellenkirchen also appears in high frequency (6 over 10) in *Rana* kl. *esculenta* of the Apetlon population.

## DISCUSSION

In metazoan organisms mtDNA appears to be maternally inherited. Evidence comes from inter and intra-specific crosses (for a review see AVISE & LANSMAN, 1983) and convincing demonstration has been reported by LANSMAN, AVISE & HUETTEL (1983) from experiments on *Heliothis* involving 91-generation crosses to males with mtDNA different to that of females. Thus mtDNA genotype of a given individual provides information about the female lineage to which it belongs. The analysis of mtDNA polymorphism among and between populations is a good tool for estimation of matriarchal phylogeny.

Also this preliminary study involves only three restriction enzymes (with a total of 4 to 7 sites per genome) noticeable discrepancies appear among the various *Rana* mtDNA patterns observed here. These DNAs can be resolved in five different morphs (LC, LA1, LA2, RP1, RP2).

The polymorphism detected also in *Rana* kl. *esculenta* and *Rana lessonae* strikingly contrasts with the absence of variation in fragment patterns within each type of mtDNA described by SPOLSKY & UZZELL (1984). An attempt to compare our results with those of SPOLSKY & UZZELL (1984) based on the length of the restriction fragments, indicates that their type A (*Rana ridibunda*) could correspond to our RP1, B (of *Rana ridibunda*) to LA1 and C (of *Rana lessonae*) to LA2. Preliminary results from experiments in progress with additional enzymes seem to confirm these homologies.

On the whole two new facts emerge.

(1) The presence within *Rana* kl. *esculenta* and *Rana lessonae* from an Austrian population of a morph (LA1) which has been found by SPOLSKY & UZZELL (1984) only in *Rana ridibunda* (type B).

These authors interpreted the low nucleotide distance between their type B (only present in *Rana ridibunda*) and their type C (only present in *Rana lessonae* and *Rana* kl. *esculenta*) as the results of an introgressive process having lead to the introduction of a *lessonae* like mitochondrial genome into a *Rana ridibunda* nuclear background. Such an hypothesis is not infirmed by our results but probably has to be modulated.

If *Rana lessonae* from Austria and *Rana ridibunda* from Poland share the same mtDNA-morph (LA1 - Type B) because of introgression, this event must have been rather recent, lacking any evolutionary divergence. Furthermore the question arises why *Rana lessonae* of Central Poland does not possess the *Rana ridibunda* morph; or what mechanism allows a fast transfer of LA1 morph from Austria to Poland (or vice versa type B from Poland to Austria)? Alternatively it may be possible that the specimens studied by SPOLSKY & UZZELL (1984), although collected in different places but all near Poznan had derived very recently from unique females (one for each type of mtDNA). This could explain the absence of variation within each type of mtDNA. Such a situation has already been proposed for some domestic species: mice (FERRIS et al., 1983), *Drosophila simulans* (BABA-AISSA & SOLIGNAC, 1984) and might also account for the homogeneity within the population of *Rana* of Chevannes. Only an analysis of mtDNA of *Rana lessonae* (and *Rana ridibunda*) from different regions between Austria and Central Poland would bring conclusive information.

(2) The identity between one of the *Rana ridibunda* mtDNA-morphs (RP1) and one of the morphs extracted from *Rana* kl. *esculenta*.

Although the number of the sites sampled is not large enough to allow a nucleotide distance calculation, this morph is clearly not very close to other morphs detected among the water frogs. Its presence in both *Rana ridibunda* and *Rana* kl. *esculenta* could be either the consequence of a recent or a past cross involving a *Rana ridibunda* female. A recent introduction of RP1 into *Rana* kl. *esculenta* from Apetlon is unlikely for several reasons. First, *Rana ridibunda* is absent from the area the hybrids came from. Second, because of the largeness of the population and the enormous frequency of *Rana* kl. *esculenta* in the entire area (TUNNER & DOBROWSKI, 1976). Third, crossing experiments suggest a rather old age of *Rana* kl. *esculenta* clones: as homotypic crosses of the hybrids, collected at Apetlon, do not give rise to viable progeny. To get more insight into the problems

concerning the origin of a *Rana ridibunda* mtDNA in the hybrid taxon further investigations are on their way. Thus, the preliminary results presented here confirm the multiplicity of the original events which lead to the appearance of *Rana* kl. *esculenta* (TUNNER, 1974; UZZELL & BERGER, 1975; TUNNER & DOBROWSKI, 1976). MtDNA studies show once more the complexity of the origin and the relationships of the western palearctic green frogs.

RESUME

Afin de préciser les relations phylogénétiques entre les trois formes: *Rana ridibunda*, *Rana lessonae* et *Rana* kl. *esculenta*, une étude de l'ADN mitochondrial d'individus récoltés à Chevannes (France), Apetlon et Prellenkirchen (Autriche), a été entreprise. Chaque ADN est analysé par électrophorèse après digestion par l'un des trois enzymes préliminairement choisis: *Bam*HI, *Eco*RI et *Pst*I. La comparaison des profils obtenus a été effectuée à trois niveaux: à l'intérieur de chaque taxon, entre les taxons vivant en sympatrie (*R. lessonae* et *R.* kl. *esculenta* de Chevannes ou d'Apetlon) et entre populations d'origine géographique différente. L'hétérogénéité de longueur, intraindividuelle, précédemment décrite chez *R.* kl. *esculenta* a été également observée chez *R. ridibunda* et *R. lessonae* quelle que soit leur origine. L'ensemble des résultats a permis d'établir cinq différentes cartes des sites de clivage (LC, RP1, RP2, LA1 et LA2). La forme LC est présente chez toutes les Grenouilles récoltées à Chevannes (*R. lessonae* et *R.* kl. *esculenta*) et leur est spécifique; deux autres (RP1 et RP2) coexistent dans la population pure de *R. ridibunda* originaire de Prellenkirchen; enfin, LA1 et LA2 apparaissent aussi bien chez *R. lessonae* que chez *R.* kl. *esculenta* d'Apetlon. Le résultat le plus important est la découverte chez *R.* kl. *esculenta* d'Apetlon de l'existence de la forme RP1 détectée chez *R. ridibunda* alors que cette dernière vit géographiquement éloignée. Il est à noter, par ailleurs, que la forme LA1 paraît semblable au génome B observé, par SPOLSKY & UZZELL, seulement chez *R. ridibunda*. L'ensemble de cette présente étude indique que les relations phylogénétiques entre les *Rana* du synklepton *esculenta* sont plus intriquées qu'il n'était originellement apparu.

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